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The role of calcium in VDAC1 oligomerization and mitochondria-mediated apoptosis☆

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ABSTRACT

The voltage-dependent anion channel (VDAC), located at the outer mitochondria membrane (OMM), mediates interactions between mitochondria and other parts of the cell by transporting anions, cations, ATP, Ca^{2+}, and metabolites. Substantial evidence points to VDAC1 as being a key player in apoptosis, regulating the release of apoptogenic proteins from mitochondria, such as cytochrome *c*, and interacting with anti-apoptotic proteins. Recently, we demonstrated that VDAC1 oligomerization is a general mechanism common to numerous apoptogens acting via different initiating cascades and proposed that a protein-conducting channel formed within a VDAC1 homo/hetero oligomer mediates cytochrome *c* release. However, the molecular mechanism responsible for VDAC1 oligomerization remains unclear. Several studies have shown that mitochondrial Ca^{2+} is involved in apoptosis induction and that VDAC1 possesses Ca^{2+}-binding sites and mediates Ca^{2+} transport across the OMM. Here, the relationship between the cellular Ca^{2+} level, $[Ca^{2+}]_i$, VDAC1 oligomerization and apoptosis was studied. Decreasing $[Ca^{2+}]_i$ using the cell-permeable Ca^{2+} chelating reagent BAPTA-AM was found to inhibit VDAC1 oligomerization and apoptosis, while increasing $[Ca^{2+}]_i$ using Ca^{2+} ionophore resulted in VDAC1 oligomerization and apoptosis induction in the absence of apoptotic stimuli. Moreover, induction of apoptosis elevated $[Ca^{2+}]_i$, concomitantly with VDAC1 oligomerization. AzRu-mediated inhibition of mitochondrial Ca^{2+} transport decreased VDAC1 oligomerization, suggesting that mitochondrial Ca^{2+} is required for VDAC1 oligomerization. In addition, increased $[Ca^{2+}]_i$ levels up-regulate VDAC1 expression. These results suggest that Ca^{2+} promotes VDAC1 oligomerization via activation of a yet unknown signaling pathway or by increasing VDAC1 expression, leading to apoptosis. This article is part of a Special Issue entitled: 12th European Symposium on Calcium.

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1. Introduction

Apart from their metabolic and apoptotic roles, mitochondria sequester Ca^{2+} at the expense of energy [1]. Mitochondria thus serve as a major hub of cellular Ca^{2+} homeostasis, fundamental for a wide range of cellular activities, such as control of oxidative phosphorylation [2,3], cell death [4–6] and secretion [7].

To regulate cytosolic and mitochondrial Ca^{2+} concentrations, mitochondria are endowed with multiple Ca^{2+} transport mechanisms located in the inner mitochondrial membrane (IMM) that mediates the uptake and release of Ca^{2+} [1]. These include the mitochondrial Ca^{2+} uniporter (MCU) [8,9], as well as its regulatory protein, the EF hand-containing protein termed MICU1 (for mitochondrial calcium uptake 1) [10]. Letm1 (leucine-zipper-EF hand-containing transmembrane region) was proposed to function in mitochondrial Ca^{2+}/H^+ exchange [11]. However, other functions, such as in mitochondrial K^+ homeostasis, have been proposed such that the contribution of Letm1 to Ca^{2+} transport has been questioned [12]. The Na^+/Ca^{2+} exchanger superfamily member NCLX serves as the major agent of Ca^{2+} efflux [13]. While all these Ca^{2+} transport systems mediate the transport of Ca^{2+} across the inner mitochondrial membrane (IMM), Ca^{2+} must first cross the outer mitochondrial membrane (OMM) before being transported across the IMM. To date, the only identified protein mediating Ca^{2+} transport in the OMM is the voltage-dependent anion channel 1 (VDAC1) [14]. Accordingly, VDAC1 is permeable to Ca^{2+} [14–16] and possesses Ca^{2+}-binding sites [14,17,18].

Located in the OMM, VDAC1 assumes a crucial position in the cell, serving as the main interface between mitochondrial and cellular metabolisms, controlling cross-talk between mitochondria and the rest of the cell [19]. VDAC1 serves as a controlled passage for anions, Ca^{2+} and other cations, adenine nucleotides and other metabolites into and out of mitochondria, thus playing a crucial role in regulating the metabolic and energetic functions of mitochondria. In addition, VDAC1 functions as an anchor point for mitochondria-interacting proteins [19] and is also recognized as a key protein in mitochondria-mediated apoptosis, participating in the release of apoptotic proteins and interacting with anti-apoptotic proteins [19].

Abbreviations: Cyto *c*, cytochrome *c*; EGS, ethylene glycol bis[succinimidylsuccinate]; OMM, outer mitochondrial membrane; PLB, planar lipid bilayer; RuR, Ruthenium red; STS, staurosporine; VDAC, voltage-dependent anion channel

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VDAC1 is highly Ca^{2+}-permeable and allows Ca^{2+} access to the mitochondrial inter-membrane space [14]. Permeability of the VDAC1 channel to Ca^{2+} was demonstrated upon reconstitution of the purified protein into a planar lipid bilayer [14,16]. In addition, La^{3+} [14], ruthenium red and AzRu [14,17,20], all of which compete with Ca^{2+} for binding sites in various proteins, are capable of inhibiting VDAC1-mediated Ca^{2+} conductivity in the lipid bilayer-reconstituted system. Finally, it was shown that Ca^{2+} regulates the uptake of Ca^{2+} into VDAC1-reconstituted liposomes [21] and that over-expression of VDAC1 in HeLa cells and skeletal myotubes enhances the transfer of Ca^{2+} into mitochondria [15].

Several lines of evidence suggest that VDAC1 possesses Ca^{2+}-binding site(s) [14,17,18]. Firstly, RuR [14], ruthenium amine binuclear complex (Ru360) [22] and AzRu, a recently-synthesized photoactivatable reagent [20], all able to specifically interact with several Ca^{2+}-binding proteins, decrease VDAC1 channel conductance in a time-dependent manner and stabilize the channel in the closed state. Such decrease in conductance can be prevented by Ca^{2+} [23], strongly suggesting that RuR and Ca^{2+} share a common binding site(s) or recognize the same VDAC1 conformation. Likewise, AzRu was also found to interact with VDAC1 and decrease channel conductance, an effect that was prevented by Ca^{2+} but not by Mg^{2+}, again suggesting interaction of AzRu with the VDAC1 Ca^{2+}-binding site(s) or a defined protein conformation [23]. RuR [17,24], like AzRu [23], had no effect on mutated E72Q- or E202Q-VDAC1 channel conductance, with [^{103}Ru]AzRu labeling native but not mutated VDAC1, suggesting that mutation of these residues stabilizes a VDAC1 conformation that doesn't bind RuR and AzRu, or alternatively, that their interaction with this conformation does not modify VDAC1 conductance.

RuR was found to protect against cell death induced by various means [24–28]. Furthermore, RuR and AzRu protected against apoptosis induced in T-REx-293 cells expressing native but not E72Q- or E202Q-mutated VDAC1 [23,24]. RuR did not interact with E72Q-VDAC1 to reduce its channel activity or protect against apoptosis in cells expressing this mutant [17]. RuR- and AzRu-mediated protection against cell death, as induced by several apoptotic stimuli [24–29], may arise from interaction with a VDAC1 Ca^{2+}-binding site or with a specific protein conformation or by inhibiting mitochondrial Ca^{2+} transport. These findings also indicate that VDAC1 functions as a Ca^{2+}-sensitive Ca^{2+} transporter in the OMM.

Mitochondrial Ca^{2+} is involved both in physiological and pathophysiological conditions [30]. Non-physiological Ca^{2+} overload depolarizes mitochondria by opening the permeability transition pore (PTP), with concomitant release of cytochrome *c* (Cyto *c*) and other IMS-located proteins, leading to both apoptotic and necrotic cell death, conditions associated with disease pathogenesis [31–33]. It is now well demonstrated that local Ca^{2+} transfer between adjacent domains of the sarco/endoplasmic reticulum (ER/SR) and mitochondria permits Ca^{2+} release from the ER/SR, leading to an enhancement of mitochondrial Ca^{2+} uptake and evoking an increase in matrix [Ca^{2+}] [34–36]. Release of Ca^{2+} from the ER via inositol-1,4,5-trisphosphate receptors (IP_3Rs) has been observed in models of apoptosis and has been directly implicated in mitochondrial Ca^{2+} overload [37]. The specific sites of physical association between the ER and mitochondria, known as mitochondria-associated membranes (MAMs), include high levels of VDAC1, among other proteins [38,39]. It has been recently established that VDAC1 (but not VDAC2 or VDAC3) selectively interacts with IP_3Rs and is preferentially involved in the transmission of low-amplitude apoptotic Ca^{2+} signals to mitochondria [40]. The involvement of VDAC1 in ER-mitochondria Ca^{2+} cross-talk places VDAC1 at a central position on the route transferring Ca^{2+} signals from the ER to the mitochondria, and thus couples ER and mitochondrial functions [40].

Although changes in mitochondrial Ca^{2+} concentration are known to trigger apoptosis, the mitochondrial target for Ca^{2+}-mediated activation of Cyto *c* release and the precise mechanism are not known. Substantial evidence, however, suggests that VDAC1 may play a role in this process.

Recently, we proposed that Cyto *c* release from the mitochondria is mediated via a central pore formed within a VDAC1 oligomeric structure, creating a pathway large enough for passage of a folded protein, such as Cyto *c*. We demonstrated that VDAC1 oligomerization is coupled to Cyto *c* release and apoptotic cell death, as induced by various stimuli [41–45]. While apoptosis inducers stimulated VDAC1 oligomerization, the apoptosis inhibitor, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), prevented STS-induced VDAC1 oligomerization and apoptosis [42,44]. In addition, VDAC1 oligomerization occurs upstream of caspase activation [42]. These results clearly indicate VDAC1 to be a component of the apoptosis machinery and support the suggestion that VDAC1 oligomerization is coupled to apoptosis induction.

In this study, the effect of alterations in cellular Ca^{2+} homeostasis on VDAC1 oligomerization and apoptosis induction was studied. We show that various apoptosis stimuli increase intracellular [Ca^{2+}] and VDAC1 oligomerization. Moreover, increasing intracellular $[Ca^{2+}]_i$ using a Ca^{2+} ionophore or thapsigargin resulted in VDAC1 oligomerization, whereas decreasing $[Ca^{2+}]_i$ using the chelator BAPTA-AM inhibited both VDAC1 oligomerization and apoptosis induction. Finally, we demonstrate that the Ca^{2+} ionophore A23187 and thapsigargin enhanced VDAC1 expression levels. The results suggest that VDAC1 oligomerization, and thus apoptosis, are regulated by cellular Ca^{2+} levels.

2. Materials and methods

2.1. Materials

Arsenic (III) oxide (As_2O_3), calcium chloride dehydrate, carboxymethyl (CM)-cellulose, *n*-decane, dimethyl sulfoxide (DMSO), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), Hepes, leupeptin, mannitol, phenylmethylsulfonyl fluoride (PMSF), propidium iodide, soybean asolectin, staurosporine (STS), sucrose, tetramethylrhodamine-methyl ester (TMRM), thapsigargin (TG) and Tris were purchased from Sigma (St. Louis, MO). Lauryl-(dimethyl)-amine oxide (LDAO) was obtained from Fluka (Buchs, Switzerland). Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad (Hercules, CA) and celite comes from Merck (Darmstadt, Germany). Coelenterazine (DeepBlueC [DBC]) was obtained from Bioline (Taunton, MA). Monoclonal anti-VDAC1 antibodies produced against the N-terminal region of 31HL human porin came from Calbiochem-Novobiochem (Nottingham, UK). Ethylene glycolbis (succinimidylsuccinate) (EGS) was obtained from Pierce. Rabbit polyclonal antibodies against VDAC1 amino acids 150–250 came from Abcam (Cambridge, UK). Monoclonal antibodies against actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated anti-mouse antibodies were obtained from Promega (Madison, WI). Annexin V (FITC) was from Enzo Life Sciences (Lausen, Switzerland), BAPTA-AM was obtained from Tocris Bioscience (Bristol, UK), Fluo-4-AM was obtained from Invitrogen (Grand Island, NY), dihydro-rhodamine-2-acetylmethyl ester (Rhod-2-AM) was from Teflabs (Austin, Texas), and siRNA was purchased from Dharmacon (Lafayette, CO). JetPRIME was from PolyPlus Transfection (Illkirch, France). Hank's balanced salts solution (HBSS) without calcium, magnesium and phenol red, Dulbecco's modified Eagle's medium (DMEM) growth media, and the supplements fetal calf serum, L-glutamine, penicillin-streptomycin, were obtained from Biological Industries (Beit Haemek, Israel).

2.2. Cell growth and transfection

T-REx-293 (transformed primary human embryonal kidney) or HeLa (human cervical adenocarcinoma) cells were maintained in DMEM supplemented with 10% fetal calf serum (1% or 0% fetal calf serum when conducting ionomycin or thapsigargin treatments, respectively), 2 mM L-glutamine, 1000 U/ml penicillin and 1 μg/ml streptomycin and maintained in a humidified atmosphere at 37 °C with 5% CO_2.

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